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Cell preparation and flow cytometry

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Natural killer cell immunotypes related to COVID-19 disease severity

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Detailed protocol

Cell preparation and staining

1. Venous blood samples were collected in heparin tubes and PBMCs isolated in a BSL-3 lab using Ficoll gradient centrifugation using SepMate from STEMCELL during 12 min at 1200g. Then cells where washed two times using PBS + FCS (2%) and centrifuged during 8 min at 400g (acc 9, dec 9).
2. Before staining, cells were counted inside the hood using an automated cell counter (Countess II, ThermoFisherÒ) after dilution using trypan blue (10mL cell suspension +10mL trypan blue).
3. PBMCs were thereafter stained fresh with the antibody mix in 96 wells plate. The antibody mix was composed by antibodies according to their titration, FACS buffer (PBS, 5mM EDTA, 1% FCS) and Brillant stain Buffer from BD. 3 mixes where done. One for extracellular stainings, one for secondary staining and one for intracellular staining.
4. Staining were done using 50uL of the mix and done during 15 minutes at room temperature in the dark. Between each stainings, cells where washed 3 times using FACS Buffer. Live/Dead cell discrimination was performed using fixable viability dye (Invitrogen). Cells were permeabilized with the Foxp3/Transcription Factor Staining Kit (eBioscience).
5. After staining, cells were fixed with 1% paraformaldehyde for 2 hours and then washed with FACS buffer before being acquired on a BD FACSymphony with 355-, 405-, 488-, 561-, and 640-nm lasers. To be sure that the 2 hours incubation in 1% paraformaldehyde did not affect the quality of the staining tests were made on blood from heathy donor by comparing the staining of the same donors with and without this step. These tests did not highlight effects of the treatment on the staining quality of the samples.
6. In addition, 50 µl of whole blood from each patient and healthy control was used with BD Trucount Tubes to obtain absolute counts of NK cells in the blood according to the instructions from the manufacturer. For each batch of experiments, PBMC from the healthy blood donor was used as internal control.

Flow cytometry data analysis

1. FCS3.0 files were exported from the FACSDiva and imported into FlowJo v.10.6.2 for subsequent analysis. The following plug-ins were used: FlowAI (2.1), DownSample (3.2), UMAP (3.1), and PhenoGraph (2.4).
2. First, the data were preprocessed using FlowAI (all checks, second fraction FR = 0.1, alpha FR = 0.01, maximum changepoints = 3, changepoint penalty = 500, and dynamic range check side = both) to remove any anomalies present in the FCS files.
3. Then, the compensation matrix for the 28-color flow cytometry panel was generated using UltraComp eBeads^{tm} from ThermoFisher SCIENTIFIC, AutoSpill and was then applied to files.
4. Dataset as such was used for the downstream analysis in both manual gating and automated analysis.
5. For the automated analysis, events were first downsampled from the NK gate across all samples using DownSample. Clinical parameter categorical values for each sample were added to downsampled populations as metadata to enable identification of these groups, and these were then concatenated for analysis. UMAP was run using all parameters from the panel except BV510 (Live/Dead, CD14, CD15, and CD19) and phycoerythrin (PE)–Cy5 (CD3).
6. PhenoGraph was run using the same parameters from the panel as UMAP (and k = 30). Fifteen thousand cells per sample were exported from the NK gate, apart from six patient samples with fewer events where all cells were taken. When assigning categorical groups formed by different clinical parameters, there was an uneven number of patients represented in each group (e.g., 17 “healthy controls,” 12 “viremic,” and 12 “nonviremic” patients). Over- and underrepresented input groups will be similarly weighted in the PhenoGraph output clusters. Therefore, we normalized the PhenoGraph output clusters to account for the total number of cells from each input group.
7. Certain figures were generated in R (versions 3.6.0 and 3.6.1) with packages factoextra (v1.0.5), RColorBrewer (v1.1-2), ggplot2 (v3.2.1 and v3.3.0), tidyr (v.1.0.2), randomcoloR (v.1.1.0.1), reshape2 (v.1.4.3), viridis (v.0.5.1), and pheatmap (v.10.12).

Strategy to identify adaptive NK cell expansions

CMV-seropositive healthy controls and COVID-19 patients displaying more than 5% of NKG2C^{+}CD57^{+} cells within their CD56^{dim} NK cell population were considered to have adaptive NK cell expansions (fig. S5A). In all individuals with adaptive expansions, adaptive NK cells displayed higher (>20%) frequencies of either CD57, CD38, or single KIRs compared with the nonadaptive NK cells (and also in one case high NKG2A).

How to cite:(Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Björkström, N. (2021). Cell preparation and flow cytometry. Bio-protocol Preprint. bio-protocol.org/prep1108.
2. Maucourant, C., Filipovic, I., Ponzetta, A., Aleman, S., Cornillet, M., Hertwig, L., Strunz, B., Lentini, A., Reinius, B., Brownlie, D., Cuapio, A., Ask, E. H., Hull, R. M., Haroun-Izquierdo, A., Schaffer, M., Klingström, J., Folkesson, E., Buggert, M., Sberg, J. K., Eriksson, L. I., Rooyackers, O., Ljunggren, H., Malmberg, K., Michaëlsson, J., Marquardt, N., Hammer, Q., Strålin, K. and Björkström, N. K.(2020). Natural killer cell immunotypes

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